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Please send me copies of the following :

1. Chong, M. W. et al., Int. J. Pharmaceutics (Jun 1998) 167(1-2): 25-36
2. Chen, B.-L. et al. J. Pharmaceutical Sciences (1994) 83(12): 1657-1661
3. Chen, B.L. et al. Pharmaceutical Res. (1994) 11(11): 1581-1587
4. Liu, H. et al. Mol. Cell. Biochem. (1997) 169(1&2): 43-50
5. Negro, A. et al. European J Biochemistry (1996) 241(2): 507-515

The first and most completely characterized neuronal action of LIF was that of promoting the transition from noradrenergic to cholinergic function in cultured sympathetic neurons (Yamamori et al., 1989). A similar switch in neurotransmitter phenotype occurs in transgenic mice overexpressing LIF in the appropriate tissue (Bamber et al., 1994). LIF influences neuropeptide expression (Patterson and Nawa, 1993; Lewis et al., 1994), and regulates mRNAs encoding muscarinic and substance P receptors in sympathetic neurons (Ludlam and Kessler, 1993; Ludlam et al., 1994). In addition, LIF promotes the survival of dorsal root ganglion sensory neurons (Murphy et al., 1993), embryonic motor neurons (Martinou et al., 1992), and postnatal sympathetic neurons (Kotzbauer et al., 1994) *in vitro*. When applied to peripheral nerves *in vivo*, LIF is retrogradely transported and rescues damaged sensory neurons (Hendry et al., 1992; Cheema

et al., 1994). Disruption of the LIF gene demonstrates a role for this cytokine in neuronal response to injury (Rao et al., 1993). Finally, LIF promotes the survival of one population of macroglial cells in culture, the oligodendrocytes (Barres et al., 1993; Mayer et al., 1994).

Injection of high doses of LIF into mice results in elevated numbers of splenic megakaryocytes and platelets, an acute phase response, splenomegaly, excess and ectopic bone formation, and gonadal dysgenesis (Metcalf and Gearing, 1989; Mayer et al., 1993). Transgenic mice overexpressing LIF displayed an altered thymic epithelium and apparent interconversion of thymic and lymph node phenotypes (Shen et al., 1994). The results complement those reported for LIF knockout mice, which show evidence of reduced myeloid progenitors and defective thymic T-cell activation (Escary et al., 1993). Levels of LIF are elevated in various inflammatory conditions (Waring et al., 1992).

The biological effects of LIF are mediated by interaction ( $K_d \approx 1$  nM) between the ligand and a specific LIF receptor subunit (LIFR $\beta$ ) that is a member of the cytokine-binding family of receptor subunits (Taga and Kishimoto, 1992; Cosman, 1993). Formation of a high-affinity signaling complex ( $K_d \approx 10$  pM) requires the association of the LIF-LIFR $\beta$  complex with another transmembrane signal transducing molecule gp130 (Gearing et al., 1992), which itself exhibits features of the cytokine family of receptors (Hibi et al., 1990).

LIF-deficient mice display T-cell defects but no evidence of obvious sensory or motor abnormalities (Escary et al., 1993), perhaps because of overlapping activities of other neurokinines, while LIFR $\beta$  is essential in motor neurons (Li et al., 1995). Understanding the roles that LIF plays in nervous system function *in vivo* would be facilitated by agents able to selectively deplete LIFR $\beta$ -bearing cell populations. One approach that has been used successfully in the case of nerve growth factor (NGF) relies on killing NGF receptor-expressing neurons by administering a saporin-NGF receptor antibody complex (Wiley, 1992; Heckers et al., 1994). Here we have constructed a diphtheria toxin (DT)-human LIF chimera, and we have shown it to be selectively cytotoxic for LIF-responsive neuronal cells *in vitro*. Further, the effects of the fusion toxin required binding to the LIF receptor, internalization by receptor-mediated endocytosis, and passage through an acidic compartment. An initial examination of the signaling actions of LIF and the DT-LIF fusion protein was also performed.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases and T4 DNA ligase were purchased from Gibco-BRL. Deoxyoligonucleotide primers were synthesized using cyanoethyl phosphoramidite chemistry and an Applied Biosystems Model 380B automated DNA synthesizer. PCR was carried out (Saiki et al., 1988) with the GeneAmp PCR reagent kit (Cetus-Perkin Elmer) according to the manufacturer's instructions. Plasmid pBBG46, which contains a synthetic gene for the mature human LIF sequence cloned in pUC18, was purchased from British BioTechnology; plasmid pRSETB was from Invitrogen. The plasmid pENKAT-12 contains human proenkephalin promoter sequences fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (Comb et al., 1986). The host cell lines *E. coli* BL21 (DE3) and BL21 (DE3) LysE were obtained from Novagen. DNA manipulation, transformation and plasmid purification were performed according to published procedures (Sambrook et al., 1989). All plasmids were constructed in *E. coli* strain HB101 (Gibco-BRL) and their DNA sequences verified by the double-stranded dideoxynucleotide chain-termination method (Sanger et al., 1977), using

the Sequenase reagent kit (United States Biochemical). Recombinant human CNTF and DT-(1-389)-Arg-Ile-Tyr-Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Met-Gly-Arg-Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Asp-CNTF-(14-200) were prepared as described by Negro et al. (1991) and Negro and Skaper (1995), respectively. Recombinant human IL-6 was purchased from British BioTechnology.

**Construction of plasmid pRSET-LIF and pT7.7-LIF.** Two oligonucleotides derived from the 5' and 3' coding regions of the human LIF gene were used as primers for amplification of the coding region by PCR, using plasmid pBBG46 as template. The forward primer contained a *Bam*HI restriction site at its 5' end. The reverse primer was positioned at the 3' end of the human LIF gene and contained an *Eco*RI restriction site. The sequences of the deoxyoligonucleotide primers were as follows:  $\alpha$  (forward): 5'-ATGGATCCGAGCCCCCTCCCCATCACCCC-3';  $\beta$  (reverse): 5'-TCGAATTCGATCCTCATTAGAAAGC-3'.

PCR was carried out using a Cetus-Perkin Elmer 480B DNA thermal cycler. After a 5 min incubation at 94°C, the first five cycles were: 94°C, 1 min; 45°C, 1 min; 72°C, 3 min; these cycles were followed by an additional 23 cycles in which the annealing temperature was 55°C. The program was terminated with a 15-min incubation at 72°C. The amplified sequences were analyzed on a 0.8% agarose gel to assess their purity, and were then cut with *Bam*HI and *Eco*RI and cloned in the same restriction sites of plasmid pRSETB to give plasmid pRSET-LIF (Fig. 1). Plasmid pT7.7-LIF was constructed by PCR as above, using a new forward deoxyoligonucleotide with the sequence:  $\gamma$  (forward): 5'-AGCATATGAGCCCCCTCCCCATCACCCC-TG-3'.

The amplified sequence was then cut with *Nde*I and *Eco*RI and cloned in the same restriction sites of plasmid pRSETB to give plasmid pT7.7-LIF.

**Construction of plasmid pDAB<sub>389</sub>-LIF and related mutants.** To clone LIF fused to DT, pRSET-LIF was cut at *Nhe*I and *Eco*RI and cloned in plasmid pTOX-CNTF (Negro and Skaper, 1995) in the same restriction sites, thereby replacing the CNTF sequences with LIF sequences to give plasmid pDAB<sub>389</sub>-LIF. Deletion of C-terminal LIF sequences from pDAB<sub>389</sub>-LIF was accomplished by cutting with either *Spe*II/*Hind*III (and refilling of the protruding ends with Klenow polymerase) to give plasmid pDAB<sub>389</sub>-LIF $\Delta$ S, or with *Stu*I/*Hind*III (and refilling of the protruding ends with Klenow polymerase) to give plasmid pDAB<sub>389</sub>-LIF $\Delta$ K. In this way, mutants were generated that lacked the last 30 or 56 C-terminal amino acids, of LIF respectively (Fig. 1).

**Synthesis and purification of recombinant proteins.** All expression plasmids constructed in *E. coli* HB101 were transferred in *E. coli* BL21 (DE3) LysE for expression of the recombinant protein. Freshly prepared bacterial cells bearing the recombinant expression plasmid were grown at 28°C in Luria-Bertani broth with 100  $\mu$ g/ml ampicillin and 30  $\mu$ g/ml chloramphenicol, until the absorbance (590 nm) of the culture suspension reached 0.6. Synthesis of the chimeric toxin was induced by adding 1 mM isopropyl thio- $\beta$ -D-galactoside. This inactivates the *lac* repressor, thereby permitting synthesis of T7 RNA polymerase which, in turn, transcribes DNA sequences next to the T7 promoter resulting in production of the fusion protein. 2 h later, bacteria were harvested by centrifugation and washed with 137 mM NaCl, 2.7 mM KCl, 9.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 (NaCl/P<sub>i</sub>). Cells were resuspended in buffer A (4 M guanidinium, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris/HCl, 0.1% Tween-20, 10 mM 2-mercaptoethanol, pH 8.0) and lysed by sonication (Branson Sonifer/power setting 6, 10 min, 4°C). The lysate was clarified by centrifugation (18000 $\times$ g, 4°C, 30 min)

and the supernatant then filtered through a 0.45- $\mu$ m membrane (Millipore). This filtrate was then loaded onto a ion metal ( $\text{Ni}^{2+}$ ) affinity chromatography (IMAC) column (0.8 cm $\times$ 4 cm) (Ni-NTA; Diagen) equilibrated in buffer A. The column was washed with buffer A at pH 7.5; the recombinant protein was eluted by lowering the buffer pH to 6.5. The recovered protein was exhaustively dialyzed at 4°C against  $\text{NaCl}/\text{P}_i$  (pH 8.3). The nomenclature for the fusion proteins derived from the various plasmids is as follows: pRSET-LIF, HIS-LIF [Met-Arg-Gly-Ser-His-His-His-His-His-Gly-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Met-Gly-Arg-Asp-Leu-Tyr-Asp-Asp-Asp-Asp-LIF-(2-184)]; pDAB<sub>389</sub>-LIF, DT-(1-389)-LIF-(2-184)-peptide [DT-(1-389)-Arg-Ile-Tyr-Met-Arg-Gly-Ser-His-His-His-His-His-Gly-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Met-Gly-Arg-Asp-Leu-Tyr-Asp-Asp-Asp-Asp-LIF-(2-184)]; pDAB<sub>389</sub>-LIF $\Delta$ S, DT-(1-389)-LIF-(2-154)-peptide; pDAB<sub>389</sub>-LIF $\Delta$ K, DT-(1-389)-LIF-(2-128)-peptide.

**Production of HIS-LIF and recombinant human LIF.** Recombinant human HIS-LIF was produced from pRSET-LIF in *E. coli* BL21 (DE3) LysE, as described above for the other recombinant proteins. Purification of HIS-LIF from bacterial inclusion bodies was carried out by the IMAC method. In the case of recombinant human LIF, the protein was treated with enterokinase (1:80) at 37°C for 18 h while still bound to the IMAC column, followed by elution and purification by reverse-phase HPLC.

**Analytical SDS/PAGE and western blot analysis.** Bacterial culture samples were analyzed by SDS/PAGE on 12% polyacrylamide (Laemmli, 1970) and stained with Coomassie brilliant blue. Proteins were electroblotted onto nitrocellulose membranes (BioRad) overnight at 2 mA constant current. The nitrocellulose sheet was incubated first with 5% (mass/vol.) BSA for 1 h, and with horse antibodies to DT (Dr Rino Rappuoli, Biocine, Siena) diluted 1:100 in  $\text{NaCl}/\text{P}_i$  containing 0.1% BSA. After three washes with 0.1% albumin/ $\text{NaCl}/\text{P}_i$ , the blot was incubated with rabbit anti-horse IgG for 1 h. After thoroughly washing the blot, immunoreactive areas were visualized with Auroprobe immunogold silver stain (Janssen), following the manufacturer's instructions.

**Protein tyrosine phosphorylation.** For immunodetection of tyrosine phosphoproteins, total proteins from  $5 \times 10^6$  control or treated SH-N-BE cells were electrophoresed on an 8% polyacrylamide gel, transferred to an Immobilon membrane (Amersham) and incubated with anti-phosphotyrosine antibody PY20H (Transduction Laboratories, Lexington, Kentucky). The immunoblot was developed with horseradish peroxidase conjugated to secondary antibody and visualized by enhanced chemiluminescence (Amersham).

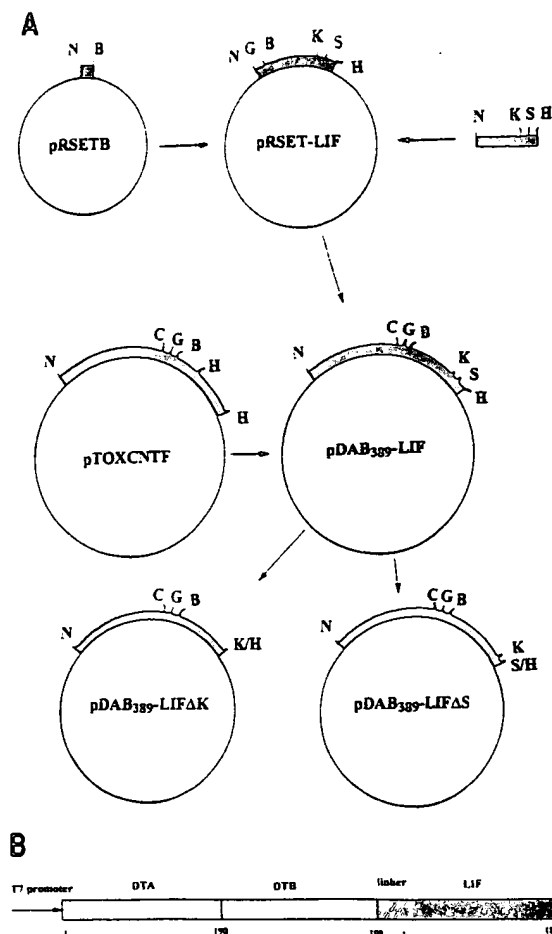
**In vitro cytotoxicity assays.** The following cell lines were used: human neuroblastomas SK-N-SH (Clone SY-5Y) (Biedler et al., 1973) and SH-N-BE (Tarroni et al., 1992); human fibroblasts, TAR; mouse embryonic stem cells, D3 (Williams et al., 1988); Chinese hamster ovary cells, CHO-K1. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal calf serum (FCS) (Gibco) at 37°C in a humidified incubator with an atmosphere of 5%  $\text{CO}_2/95\%$  air. For cytotoxicity assays, cells were seeded in 96-well plates ( $10^4$  cells/well) in culture medium. Fusion proteins (100 nM to 1 pM) in medium were then added to the culture and the incubation continued for 40 h. Inhibition assays were performed using recombinant human CNTF, LIF, or IL-6. Control cultures contained medium alone. After this time, the medium was exchanged for 200  $\mu$ l leucine-free medium containing 1  $\mu\text{Ci}/\text{ml}$  [ $1\text{-}^{14}\text{C}$ ]leucine (New England Nuclear). After incubation for a further 90 min, medium was removed and the cells were lysed by adding 60  $\mu$ l 0.4 M KOH/well. Proteins were pre-

cipitated with 10% trichloroacetic acid (140  $\mu$ l/well) and insoluble material collected on glass fiber filters (GFA; Whatman) under vacuum. The filters were washed with 5% trichloroacetic acid, dried, and the radioactivity was measured by standard liquid scintillation methods. Concentration/response curves were constructed by comparing the percentage decrease in [ $^{14}\text{C}$ ]leucine incorporation in cultures treated with the fusion toxin relative to untreated controls.

**Neurotoxicity.** DT-(1-389)-LIF-(2-184)-peptide was assayed for its cytotoxic effects on neurons cultured from chicken embryonic day 10-11 lumbosacral sympathetic ganglia. Cells were prepared as described previously, with a 3 h preplating step over plastic to enrich the neuronal population (Skaper et al., 1990). Microwell plates (6-mm diameter) coated with polyornithine and laminin were seeded with 2000 neurons/well in culture medium (Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 10% fetal calf serum (FCS) and 50 ng/ml mouse 2.5S NGF). 48 h later, 50% of the medium in the wells was exchanged for 50  $\mu$ l fresh medium (NGF-free) containing various dilutions of DT-(1-389)-LIF-(2-184)-peptide. Survival was assessed 72 h later by counting over two diametrical strips (20% of the total surface area) the number of neurons having neurites greater than two somal diameters in length.

**Measurement of c-fos and proenkephalin mRNAs.** Neuroblastoma SH-N-BE cells were shifted to medium with 0.5% FCS 24 h prior to treatment to minimize possible contributions of serum components. Cells were exposed to 20 ng/ml recombinant human LIF or 80 ng/ml of DT-(1-389)-LIF-(2-184)-peptide in medium with 0.5% FCS for various times. Cellular RNA was then extracted using the acid guanidinium thiocyanate/phenol/chloroform procedure (Chomczynski and Sacchi, 1987). 20  $\mu$ g total RNA was separated by electrophoresis on a 1.2% agarose gel, followed by capillary blotting onto nylon membranes (Hybond-N<sup>+</sup>, Amersham). The blot was then hybridized with one of the following: a 0.8-kb *SphI*-*SalI* fragment of mouse *c-fos* (Gabellini et al., 1991); a 0.7-kb rat proenkephalin cDNA fragment (Yoshikawa et al., 1984); a 1.2-kb *PstI* fragment of glyceraldehyde-3-phosphate dehydrogenase (*GAP-DH*) as internal control (Fort et al., 1985). All probes were radio-labelled with [ $\alpha\text{-}^{32}\text{P}$ ]dCTP using a random primer labelling kit (Amersham). Hybridization was carried out for 24 h at 42°C in 50% formamide, 5% sodium dextran sulphate, 5 $\times$ Denhardt's solution and 750 mM NaCl, 75 mM sodium citrate, pH 7.0 (Sambrook et al., 1989). After washing under high stringency conditions, the blots were exposed for 2-5 days at -70°C to hyperfilm (Amersham MP) with intensifying screens, and the film then developed.

**DNA transfection.** Neuroblastoma SH-N-BE cells were transfected using a commercially available liposome transfection reagent (DOTAP, Boehringer-Mannheim). Briefly, 10  $\mu$ g plasmid pENKAT-12 (Comb et al., 1986) or 10  $\mu$ g plasmid pBSFC-series, the latter containing the bacterial *CAT* gene under the control of the mouse *c-fos* promoter (Gabellini et al., 1991), in 100  $\mu$ l 100 mM Hepes, (pH 7.4) was added to 100  $\mu$ l 40% liposome solution (DOTAP). After 15 min, this mixture was added to the cells, and incubation carried out for 8 h (37°C). The transfection solution was then removed and the cells were maintained for 24 h in medium with 0.5% FCS. A construct containing the luciferase gene (pCMV-luciferase), under the control of the CMV promoter/enhancer element, was co-transfected with the pENKAT-12 and pBSFC-series plasmids. pCMV-luciferase is not stimulated by LIF (data not shown). *CAT* protein activity was quantified with a *CAT*-ELISA kit (Boehringer-Mannheim), and was expressed relative to the percentage induction of control plasmid pSV40-luciferase. The *c-fos*-*CAT* plasmids pBSFC-459



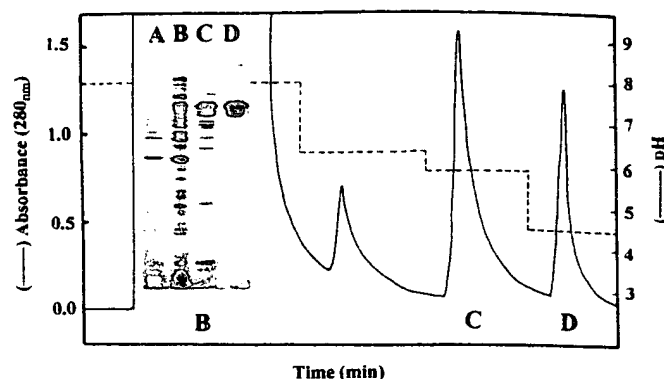
**Fig. 1.** Plasmids used in the construction of DT-(1-389)-LIF-(2-184)-peptide and derivative fusion toxins. (A) Plasmid pDAB<sub>389</sub>-LIF carries the hybrid gene encoding the first 389 amino acids of DT fused to human LIF (lacking the first N-terminal amino acid) through a bridge spanning 34 amino acids. Restriction endonuclease sites: B, *Bam*HI; C, *Cla*I; G, *Nhe*I; H, *Hind*III; K, *Kpn*I; N, *Nde*I; S, *Spe*I. K/H and S/H represent the positions of C-terminal deletions corresponding to DT-(1-389)-LIF-(2-154)-peptide and DT-(1-389)-LIF-(2-128)-peptide, respectively. See Materials and Methods section for further details. (B) A linear map of DT-(1-389)-LIF-(2-184)-peptide is shown. The position and direction of the T7 promoter for expression of recombinant protein is indicated. DTA and DTB represent domains A and B of diphtheria toxin, with the linker between the toxin and LIF.

and pBSFC-307, which carry selected deletion mutants of the *c-fos* promoter, have been described (Gabellini et al., 1991). Plasmids pBSFC-358, pBSFC-325, and pBSFC-143 were derived from pBSFC-459. The latter three plasmids were constructed by amplifying *c-fos* promoter sequences of plasmid pc-FOS (mouse-3) (ATTC41041), with adapted primers utilizing PCR and replacing the *fos* sequences of plasmid pBSFC-459. All plasmids are designated by the number of residual nucleotides from the transcription start site.

## RESULTS

### Expression and purification of a chimeric DT-LIF protein.

The structural gene encoding DT-(1-389)-LIF-(2-184)-peptide was assembled using a strategy similar to that described for a DT-related CNTF fusion protein (Negro and Skaper, 1995) (Fig. 1). In the present construct, a 34-amino-acid span was inserted between the sequences for DT and LIF (Fig. 1), and in-



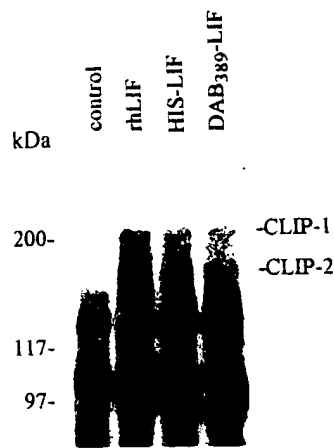
**Fig. 2.** Immobilized metal ion affinity column purification of DT-(1-389)-LIF-(2-184)-peptide. Elution profile from a Ni<sup>2+</sup>-IMAC column of the extract from pDAB<sub>389</sub>-LIF-expressing cells. Inset: SDS/PAGE analysis (10% polyacrylamide) of fractions pooled from the indicated protein peaks. Lane A, crude extract from bacterial cells carrying plasmid pDAB<sub>389</sub>-LIF; lane B, peak B fractions; lane C, peak C fractions; lane D, peak D fractions. DT-(1-389)-LIF-(2-184)-peptide eluted in peak D as a single 66-kDa band by SDS/PAGE (10% polyacrylamide).

cluded six consecutive histidine residues to allow for binding to an IMAC column (Hochuli et al., 1988).

The LIF gene is toxic for *E. coli* BL21 (DE3) cells. Hence, all plasmids were cloned in *E. coli* BL21 (DE3) LysE, instead of in *E. coli* BL21 (DE3) as was the case for pTOXCNTF (Negro and Skaper, 1995). *E. coli* BL21 (DE3) LysE contains a plasmid carrying a gene for lysozyme whose expression is a strong inhibitor of T7 RNA polymerase, making it possible to place in *E. coli* BL21 (DE3) LysE cells a plasmid expressing a toxic gene (Studier et al., 1990). T7 RNA polymerase repression was, however, incomplete, as colonies of *E. coli* BL21 (DE3) LysE transformed with plasmid pDAB<sub>389</sub>-LIF were about 25% the size of those produced by the same bacterial cells transformed with pTOXCNTF (Negro and Skaper, 1995). Cytotoxicity was more pronounced when *E. coli* BL21 (DE3) LysE was transformed with plasmid pRSET-LIF (yielding HIS-LIF), and, in particular, when *E. coli* BL21 (DE3) LysE cells were transformed with a plasmid carrying the gene for recombinant human LIF (yielding Met-LIF). This latter cytotoxicity was abolished when either the last 30 or 56 amino acids of the LIF sequence were deleted (plasmids pDAB<sub>389</sub>-LIFΔS and pDAB<sub>389</sub>-LIFΔK, respectively), which directly implicates the LIF domain.

The expression level of fusion protein was inversely proportional to its relative cytotoxic activity. DT-(1-389)-LIF-(2-184)-peptide from induced *E. coli* cells was readily identified on SDS/PAGE gels, accounting for about 7% of total bacterial protein. HIS-LIF was expressed at a level below 1% of total protein and was detectable by SDS/PAGE only after purification. LIF was only weakly visible in extracts of induced *E. coli* carrying the LIF expression plasmid. All fusion proteins cross-reacted with polyclonal antibodies to diphtheria toxin (data not shown).

DT-(1-389)-LIF-(2-184)-peptide accumulated in inclusion bodies, as was expected for recombinant protein expression in *E. coli*. The incorporation of six consecutive histidine residues in the fusion protein permitted a single-step purification by means of a Ni<sup>2+</sup> resin affinity column (Fig. 2). The use of guanidinium/2-mercaptoethanol aided both protein solubilization and refolding on the affinity column. Proteins containing disulphide bridges are commonly refolded at low concentrations to minimize possible intermolecular bridge formation. Retention of DT-(1-389)-LIF-(2-184)-peptide by the IMAC resin elimi-



**Fig. 3.** DT-(1-389)-LIF-(2-184)-peptide, recombinant human LIF and HIS-LIF induce tyrosine phosphorylation of CLIP1 and CLIP2. SH-N-BE cells ( $5 \times 10^6$ ) were incubated for 30 min with 100 ng/ml recombinant human LIF, 100 ng/ml HIS-LIF or 200 ng/ml DT-(1-389)-LIF-(2-184)-peptide (DAB<sub>389</sub>-LIF). Cell proteins were separated by SDS/PAGE (5% polyacrylamide), blotted and incubated with anti-phosphotyrosine antibodies.

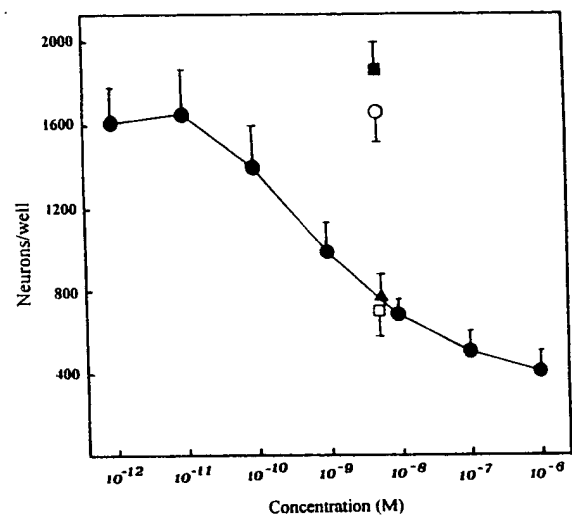
nated the risk of aggregation and permitted its elution at relatively high concentrations. Fusion proteins isolated in this way have a final purity above 95%. Typically, 1 mg of pure refolded DT-(1-389)-LIF-(2-184)-peptide could be obtained per liter of *E. coli* broth. The molecular masses of the various recombinant proteins were in good agreement with their anticipated relative molecular masses, as deduced from the nucleic acid sequence of the corresponding structural gene.

**Protein tyrosine phosphorylation.** Importantly, DT-(1-389)-LIF-(2-184)-peptide, like LIF, directly activated the LIF receptor complex in SH-N-BE neuroblastoma cells. Incubation of SH-N-BE cells with 100 ng/ml recombinant human LIF, 100 ng/ml HIS-LIF, or 200 ng/ml DT-(1-389)-LIF-(2-184)-peptide for 30 min led to the tyrosine phosphorylation of the LIF receptor-associated proteins CLIP1 and CLIP2 (Ip et al., 1992) (Fig. 3).

**Cytotoxicity of DT-(1-389)-LIF-(2-184)-peptide.** Several LIF-receptor-positive and LIF-receptor-negative cell lines were tested for their sensitivity to DT-(1-389)-LIF-(2-184)-peptide by means of a [ $^3$ H]leucine uptake assay. The human neuroblastoma cell line SY-5Y expresses gp130 (Ip et al., 1992) and is CNTF-responsive (Squinto et al., 1990). SH-N-BE, a clonal derivative of SY-5Y, is also expected to contain an intact LIF receptor complex, as these cells are responsive both to CNTF (Negro and Skaper, 1995; Rossino et al., 1995) and to LIF (present study). LIF is able to maintain mouse embryonic stem cells D3 in an undifferentiated state, these cells having been used to produce transgenic mice with ablated genes via homologous recombination (Gearing et al., 1989). The human fibroblast line TAR and Chinese hamster ovary KI cells (CHO-KI) were used as negative controls, as they lack LIFR $\beta$ . Cells SY-5Y, SH-N-BE, and D3 were highly sensitive to the cytotoxic action of DT-(1-389)-LIF-(2-184)-peptide ( $IC_{50} \approx 1-10$  nM), whereas cells having no detectable levels of LIF receptors were more resistant to DT-(1-389)-LIF-(2-184)-peptide-mediated toxicity ( $IC_{50} > 100$  nM) (Table 1). Further, LIF- and CNTF-responsive sympathetic ganglionic neurons were killed by a 3-day exposure to DT-(1-389)-LIF-(2-184)-peptide ( $LC_{50}$  0.9 nM) (Fig. 4). The C-terminal variant fusion toxins DT-(1-389)-LIF-(2-154)-peptide and DT-(1-389)-LIF-(2-128)-peptide were ineffective on

**Table 1.** Comparative cytotoxicity of DT-(1-389)-LIF-(2-184)-peptide and its variants for eukaryotic cell lines.  $IC_{50}$ , concentration of DT-(1-389)-LIF-(2-184)-peptide that inhibits protein synthesis in target cells by 50% after a 40-h exposure. Values are means from three independent experiments. Standard deviations were within 5% of the respective means. n.d., not determined

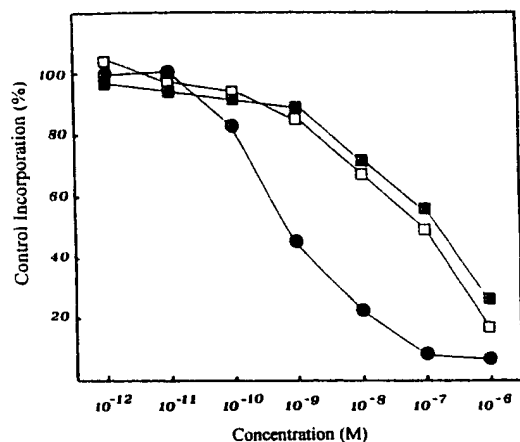
Cell line	$IC_{50}$		
	DT-(1-389)-LIF-(2-184)-peptide	DT-(1-389)-LIF-(2-154)-peptide	DT-(1-389)-LIF-(2-128)-peptide
	nM		
SH-N-BE	1.1	>100	>100
SY-5Y	9	>100	>100
DE3	80	>100	>100
TAR	>100	n.d.	n.d.
CHO-KI	>100	>100	>100



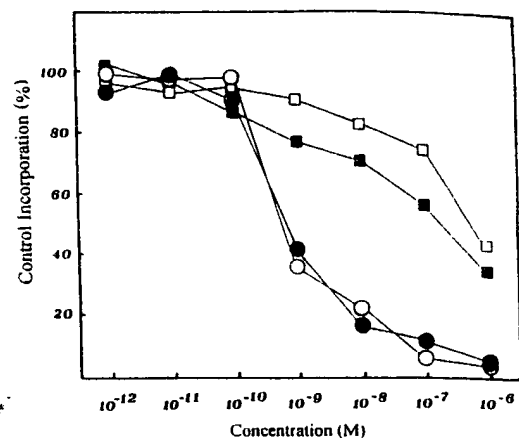
**Fig. 4.** DT-(1-389)-LIF-(2-184)-peptide fusion protein is cytotoxic for sympathetic neurons. Chicken embryonic day 10-11 sympathetic neurons were plated (2000/well) in 6-mm diameter microwell plates in medium containing 50 ng/ml mouse NGF. After 48 h, one-half of the medium volume was exchanged for an equal volume of fresh medium containing twofold concentrated solutions of DT-(1-389)-LIF-(2-184)-peptide (●), to give the indicated final concentrations of the fusion toxin. In some cases, DT-(1-389)-LIF-(2-184)-peptide (5 nM) was added, alone (□) or together with a 50-fold excess of recombinant human LIF (○), recombinant human CNTF (■), or recombinant human IL6 (▲). All cultures were fixed after an additional 3 days incubation and surviving neurons counted. Values are means  $\pm$  SD ( $n = 8$ , two experiments). The NGF-only cultures (control) contained  $1626 \pm 294$  neurons/well when fixed.

SY-5Y, SH-N-BE and D3 cells (Table 1). Data for SH-N-BE neuroblastoma cells are shown in Fig. 5. The last result suggests that the predicted C-terminal helix D of LIF (Bazan, 1991) may be needed to render the fusion protein efficacious, although a lower binding efficiency for these mutants cannot be excluded.

That the cytotoxic effects of DT-(1-389)-LIF-(2-184)-peptide depended on LIF receptor expression were further demonstrated by competition analysis. The addition of excess recombinant human LIF (whose bipartite receptor is contained within the tripartite CNTF receptor complex) (Ip et al., 1992; Davis et al., 1993) or recombinant human CNTF to SH-N-BE cells



**Fig. 5. Incorporation of [ $^{14}$ C]leucine into SH-N-BE cells exposed to DT-(1-389)-LIF-(2-184)-peptide or variants.** SH-N-BE cells were incubated with DT-(1-389)-LIF-(2-184)-peptide (●), DT-(1-389)-LIF-(2-154)-peptide (■), or DT-(1-389)-LIF-(2-128)-peptide (□). Cytotoxicity assays were performed as described in the Materials and Methods section. The percentage incorporation of [ $^{14}$ C]leucine relative to untreated controls was determined in each case. Values are means of three experiments with the standard deviation never exceeding 10%.



**Fig. 6. Incorporation of [ $^{14}$ C]leucine in SH-N-BE cells after exposure to DT-(1-389)-LIF-(2-184)-peptide: effect of competitors.** SH-N-BE cells were incubated with DT-(1-389)-LIF-(2-184)-peptide alone (○) or together with 10  $\mu$ M of one of the following: recombinant human LIF (□), recombinant human CNTF (■), recombinant human IL-6 (○). Cytotoxicity assays were performed as described in the Materials and Methods section. The percentage incorporation of [ $^{14}$ C]leucine relative to untreated controls was determined in each case. Values are means of three experiments with the standard deviation never exceeding 10%.

effectively blocked the cytotoxic action of DT-(1-389)-LIF-(2-184)-peptide, which increased the apparent  $IC_{50}$  values by approximately 1000-fold and 100-fold, respectively (Fig. 6). Recombinant human IL-6, which utilizes the signal transducing protein gp130, was, however, ineffective. A similar profile was observed for the neurotoxic effects of DT-(1-389)-LIF-(2-184)-peptide toward sympathetic neurons (Fig. 4).

Fragment A of native DT is known to be internalized by receptor-mediated endocytosis, and acidification of the endocytotic vesicles is an essential step in the intoxication process (Moskang et al., 1991). Agents (e.g. chloroquine) that prevent acidification of the endosome block DT action (Papini et al., 1993). To verify that delivery of fragment A from DT-(1-389)-LIF-(2-184)-peptide to the cytosol of target cells followed a similar route of entry, the effect of chloroquine on DT-(1-389)-LIF-(2-184)-peptide-mediated intoxication was examined. Chloroquine (10  $\mu$ M) prevented the action of the LIF fusion toxin towards SH-N-BE cells (Table 2). It thus appears that DT-(1-389)-LIF-(2-184)-peptide requires passage through an acidic vesicle to deliver its ADP-ribosyltransferase to the cytosol.

**Effects of LIF and LIF fusion toxin on *c-fos* and enkephalin gene expression.** LIF, along with a number of growth factors, up-regulates the immediate early gene *c-fos* in neuroblastoma cells (Squinto et al., 1990). This event likely results from an action of second messengers directly at the level of the *c-fos* promoter (Bonni et al., 1994). Incubation of SH-N-BE cells with 5 nM recombinant human LIF or 10 nM DT-(1-389)-LIF-(2-184)-peptide caused a time-dependent increase in *c-fos* mRNA expression; this increase was maximal (sixfold) after 30 min and subsequently declined (Fig. 7A). Recombinant human LIF and HIS-LIF also induced CAT reporter gene expression in SH-N-BE cells transfected with the *c-fos* promoter deletion constructs pBSFC-459, pBSFC-358, pBSFC-325, and pBSFC-307 while DT-(1-389)-LIF-(2-184)-peptide failed to do so, indicating that the LIF fusion toxin blocked protein synthesis (Fig. 8).

Recombinant human LIF induced, in a time-dependent and concentration-dependent manner the expression of proenkephalin mRNA in SH-N-BE cells (Fig. 7B). Unlike for *c-fos* mRNA, recombinant human LIF produced a maximum induction in pro-

**Table 2. Inhibition of DT-(1-389)-LIF-(2-184)-peptide cytotoxicity by chloroquine.** SH-N-BE cells were incubated with DT-(1-389)-LIF-(2-184)-peptide in the presence or absence (control) of chloroquine (10  $\mu$ M) for 20 h and labeled with [ $^{14}$ C]leucine to determine the level of protein synthesis. Values are means of three experiments, with the standard deviation never exceeding 10% of the mean.

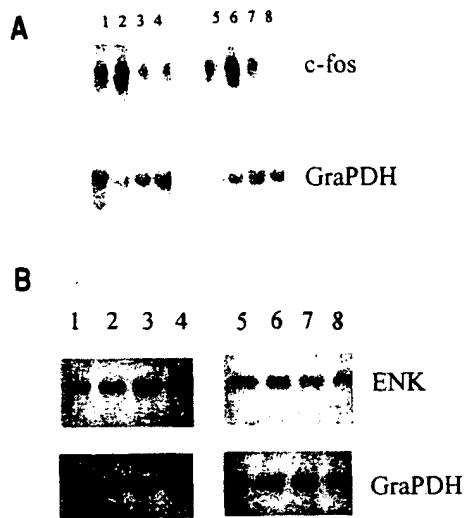
DT-(1-389)-LIF-(2-184)-peptide nM	Incorporation	
	control	chloroquine
	% control	
30	20	50
10	45	75
1	95	100

enkephalin mRNA after 6 h, which suggests that the signal transduction process was second messenger mediated. Incubation of SH-N-BE cells with DT-(1-389)-LIF-(2-184)-peptide failed to increase proenkephalin mRNA levels, further supporting a dependence of enkephalin gene expression on *de novo* protein synthesis. In addition, recombinant human LIF, but not DT-(1-389)-LIF-(2-184)-peptide up-regulated expression of the enkephalin-CAT fusion gene (maximal stimulation  $\geq 40$  ng/ml) (Fig. 8).

## DISCUSSION

LIF is a secreted polyfunctional cytokine that elicits a diverse array of biological effects on many cell types, including neurons, hepatocytes, embryonic stem cells, primordial germ cells, and adipocytes (Metcalf, 1992; Patterson, 1994). LIF, CNTF, IL-6 and oncostatin M have been linked through the similarities of their tertiary structures (Bazan, 1991) and through their interaction with related receptor complexes containing a common signal-transducing element, gp130 (Stahl and Yancopoulos, 1993). Together with LIF, these cytokines serve a wide range of overlapping activities involving hematopoietic, immunologic, and neuronal plasticity (Miyajima et al., 1992).

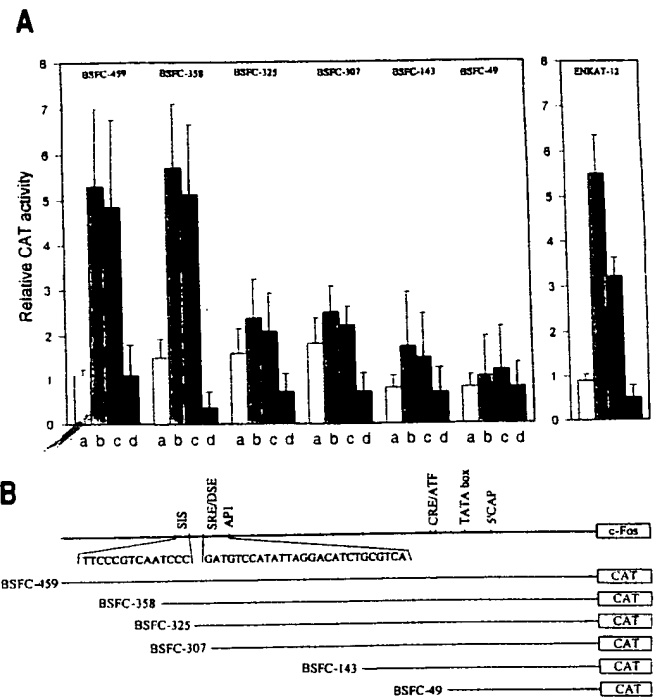




**Fig. 7.** DT-(1-389)-LIF-(2-184)-peptide and LIF increase *c-fos* mRNA but only LIF induces enkephalin expression in SH-N-BE cells. (A) SH-N-BE cells were treated with 30 ng/ml recombinant human LIF (lanes 1-4) or 100 ng/ml DT-(1-389)-LIF-(2-184)-peptide (lanes 5-8). Time 0 (lanes 1 and 5), 30 min (lanes 2 and 6), 1 h (lanes 3 and 7), 2 h (lanes 4 and 8). Densitometric assay revealed a fivefold increase of *c-fos* mRNA after 30 min in both recombinant human LIF- and DT-(1-389)-LIF-(2-184)-peptide-treated cells. (B) SH-N-BE cells were treated as above with recombinant human LIF (lanes 1-4) or DT-(1-389)-LIF-(2-184)-peptide (lanes 5-8). Time 0 (lanes 1, 5), 2 (lanes 2, 6), 6 (lanes 3, 7), 12 h (lanes 4, 8). Densitometric assay revealed a threefold increase of proenkephalin mRNA after 6 h in recombinant human LIF-treated cells only. Values were normalized to the quantity of mRNA for *GraPDH*. Similar results were obtained in two other experiments.

Here, we report the construction of a chimeric gene that encodes the DT-LIF fusion protein DT-(1-389)-LIF-(2-184)-peptide, in which the receptor-binding domain of DT is replaced with human LIF. A similar strategy has been used in producing other cytokine fusion toxins (Jean and Murphy, 1991; Negro and Skaper, 1995). A linker of 34 amino acids containing six consecutive histidine residues facilitated purification of DT-(1-389)-LIF-(2-184)-peptide by immobilized metal ion affinity chromatography, and allowed for refolding of the fusion protein while still bound to the IMAC column. The six cysteine residues in DT-(1-389)-LIF-(2-184)-peptide (two in DT and four in LIF) thus formed correct intermolecular pairs, avoiding the problem of intramolecular disulfide bonding and aggregation that frequently occurs when recombinant proteins are refolded in solution.

As for keratinocyte growth factor (Ron et al., 1993), the expression of LIF in *E. coli* was cytotoxic. Attenuated toxicity was observed for expression of the fusion toxin, probably due to its storage within inclusion bodies. In contrast, bacterial expression of the structurally related cytokines CNTF and IL-6 is not associated with such cytotoxic activities (Negro and Skaper, 1995; Negro, A., unpublished results). LIF has also been synthesized in *E. coli* as an inactive glutathione *S*-transferase fusion protein, where the biologically active protein was liberated by thrombin cleavage of the fusion protein bound to a glutathione-Sepharose column (Gearing et al., 1989). The presence of glutathione *S*-transferase in this fusion protein (Gearing et al., 1989) may have attenuated its cytotoxicity, much like for DT-(1-389)-LIF-(2-184)-peptide. Such cytotoxicity reduces the level of protein synthesis, with plasmid pRSET-LIF yielding only 3-4% HIS-LIF, as compared to a yield of 15% HIS-CNTF from plasmid pRSET-CNTF (Negro and Skaper, 1995). Expression levels did not



**Fig. 8.** Modulation by recombinant human LIF, HIS-LIF and DT-(1-389)-LIF-(2-184)-peptide of CAT activity in SH-N-BE neuroblastoma cells transfected with pFOS-CAT deletion constructs or pENKAT-12. (A) Schematic representation of the nucleotide sequence of a portion of the *c-fos* promoter. The sequence between positions -343 and -328 confers SIS binding elements; the sequence between positions -312 and -286 confers serum inducibility (SRE) and contains a dyad symmetric element with the adjacent AP-1 binding site. The major cyclic AMP responsive element (CRE/ATF) that occurs between positions -64 and -59 is also shown. Plasmids containing deleted regions of the *c-fos* promoter were termed BSFC, followed by the number of residual residues from the CAP site. (B) Induction of CAT activity after 24 h in cells transfected with the indicated constructs (shown above columns): untreated (a); plus 20 ng/ml recombinant human LIF (b); plus 45 ng/ml HIS-LIF (c); plus 300 ng/ml DT-(1-389)-LIF-(2-184)-peptide (d). Transfection efficiencies in all cases are given with respect to the activity of the cotransfected SV40-luciferase gene. All values are expressed relative to the induction (1.0) in untransfected cells. Data represent means  $\pm$  SD from duplicate cultures in each of three experiments.

change significantly for *E. coli* strains BL21 (DE3) and BL21 (DE3 LysE). An analogous construct for HIS-IL-6 transformed in BL21 (DE3) produced a yield of the recombinant protein equal to 25% of total bacterial protein (Negro, A., unpublished results).

DT-(1-389)-LIF-(2-184)-peptide directly activated the LIF receptor complex in a manner similar to LIF and selectively killed LIF-responsive sympathetic neurons, LIF receptor-bearing neuroblastoma cells, and D3 embryonic stem cells. Although the mouse *LIF* gene has been cloned (Mereau et al., 1993), the human *LIF* gene was used in the present fusion protein construct, as human LIF is also active on mouse cells (Moreau et al., 1988) while the mouse protein is less active on human cells (Owczarek et al., 1993). Deletions at the C-terminus of the LIF sequence in DT-(1-389)-LIF-(2-184)-peptide abolished its cytotoxic action. Removal of the last 30 amino acids [DT-(1-389)-LIF-(2-154)-peptide] eliminates helix D of the LIF protein (Bazan, 1991), a domain implicated in LIF receptor binding (Robinson et al., 1994). The cytotoxic effects of DT-(1-389)-LIF-(2-184)-peptide for sympathetic neurons and SH-N-BE neuroblastoma cells were inhibited by recombinant human LIF and CNTF, but



not by recombinant human IL-6. In contrast, cytotoxicity of the CNTF chimera DT-(1-389)-CNTF-(14-200)-peptide was only partially inhibited by recombinant human LIF, and not at all by recombinant human IL-6 (Negro and Skaper, 1995). These behaviors may be due to the fact that SH-N-BE cells express large quantities of CNTFR $\alpha$  (Rossino et al., 1995) and are consistent with the observed fivefold smaller IC<sub>50</sub> of DT-(1-389)-CNTF-(14-200)-peptide compared to that of DT-(1-389)-LIF-(2-184)-peptide. The antagonism by recombinant human LIF and CNTF against DT-(1-389)-LIF-(2-184)-peptide was not surprising, given that both neurokines make use of LIFR $\beta$  and gp130 to generate a signal-transducing complex. The differential potencies between recombinant human CNTF and LIF in inhibiting DT-(1-389)-LIF-(2-184)-peptide cytotoxicity could result from the following: an additional component may be required for signaling by LIF; LIF may have a higher affinity for LIFR $\beta$  than CNTF; or CNTF may be sequestered by CNTFR $\alpha$  without binding to LIFR $\beta$ . This last possibility cannot be excluded because CNTFR $\alpha$  is anchored to cell membranes by a labile glycosylphosphatidylinositol linkage (Davis et al., 1991). The inability of recombinant human IL-6 to antagonize the cytotoxicity of DT-(1-389)-LIF-(2-184)-peptide (present study) and DT-(1-389)-CNTF-(14-200)-peptide (Negro and Skaper, 1995) may be due to a lack of IL-6R expression by SH-N-BE cells (Negro, A., unpublished results).

Expression of the immediate early gene *c-fos* in SH-N-BE cells was induced by recombinant human LIF, HIS-LIF, and DT-(1-389)-LIF-(2-184)-peptide. Recombinant human LIF and HIS-LIF, but not DT-(1-389)-LIF-(2-184)-peptide, stimulated expression of a *CAT* reporter gene linked to the *c-fos* promoter in transiently transfected cells, which indirectly demonstrates that the DT-(1-389)-LIF-(2-184)-peptide inhibits protein synthesis.

An understanding of brain functions, which depend on neuron/neuron interactions, has progressed with the development of methods that permit the selective elimination of neuronal types with particular identities. For example, an immunotoxin against the p75 NGF receptor (192 IgG-saporin) (Wiley, 1992) given intraventricularly or intraparenchymally, has been used to induce a complete and selective loss of NGF receptor-positive cholinergic neurons projecting to the neocortex and hippocampus (Heckers et al., 1994). The effect of 192 IgG-saporin seemed to be mediated by terminal uptake and retrograde transport to the soma, as demonstrated by the protective action of colchicine (Heckers et al., 1994). DT-(1-389)-LIF-(2-184)-peptide may likewise find application in selectively destroying LIFR $\beta$ -expressing cells in the nervous system. When applied to peripheral nerves *in vivo*, LIF is retrogradely transported and rescues damaged sensory neurons (Hendry et al., 1992; Cheema et al., 1994). It is thus not unreasonable to expect that DT-(1-389)-LIF-(2-184)-peptide would be efficacious in ablating LIF-responsive neurons, in a manner analogous to that of the NGF receptor-target toxin. *In vivo* experiments are now planned to address this possibility.

## REFERENCES

- Bamber, B. A., Masters, B. A., Hoyle, G. W., Brinster, R. L. & Palmiter, R. D. (1994) Leukemia inhibitory factor induces neurotransmitter switching in transgenic mice. *Proc. Natl Acad. Sci. USA* 91, 7839-7843.
- Barres, B. A., Schmid, R., Sendtner, M. & Raff, M. C. (1993) Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development* 118, 283-295.
- Bazan, J. F. (1991) Neurotrophic cytokines in the hematopoietic fold. *Neuron* 7, 197-208.
- Biedler, J. L., Helson, L. & Spengler, B. A. (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* 33, 2643-2652.
- Bonni, A., Frank, D. A., Schindler, C. & Greenberg, M. E. (1994) Characterization of a pathway for ciliary neurotrophic factor signaling to the nucleus. *Science* 262, 1575-1579.
- Cheema, S. S., Richards, L., Murphy, M. & Bartlett, P. F. (1994) Leukemia inhibitor factor prevents the death of axotomized sensory neurons in the dorsal root ganglia. *J. Neurosci. Res.* 37, 213-218.
- Chomczynski, P. & Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Comb, M., Bimberg, N. C., Seasholtz, A., Herbert, E. & Goodman, H. M. (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* 323, 353-356.
- Cosman, D. (1993) The hematopoietic receptor superfamily. *Cytokine* 5, 95-106.
- Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P. & Yancopoulos, G. D. (1991) The receptor for ciliary neurotrophic factor. *Science* 253, 59-63.
- Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. & Yancopoulos, G. D. (1993) LIFR $\beta$  and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* 260, 1805-1808.
- Escary, J.-L., Perreau, J., Duménil, D., Ezine, S. & Brûlet, P. (1993) Leukaemia inhibitor factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature* 363, 361-364.
- Fort, P., Marty, M., Piechaczyk, M., El Sabrouy, S., Dani, C., Jeanteur, P. & Blanchard, J. M. (1985) Various rat tissues express only the major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase multigenic family. *Nucleic Acids Res.* 13, 1431-1442.
- Fukuda, K. (1985) Purification and partial characterization of a cholinergic differentiation factor. *Proc. Natl Acad. Sci. USA* 82, 8795-8799.
- Gabellini, N., Facci, L., Milani, D., Negro, A., Callegaro, L., Skaper, S. D. & Leon, A. (1991) Differences in induction of *c-fos* transcription by cholera toxin-derived cyclic AMP and Ca<sup>2+</sup> signals in astrocytes and 3T3 fibroblasts. *Exp. Cell. Res.* 194, 210-217.
- Gearing, D. P., Nicola, N. A., Metcalf, D., Foote, S., Tracy, A., Gough, N. N. & Williams, L. R. (1989) Production of leukemia inhibitor factor in *Escherichia coli* by a novel procedure and its use in maintaining embryonic stem cells in culture. *Biotechnology* 7, 1157-1161.
- Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F. & Cosman, D. (1992) The IL-6 transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* 255, 1434-1437.
- Hall, A. K. & Rao, M. S. (1992) Cytokines and neurokines: related ligands and receptors. *Trends Neurosci.* 15, 35-37.
- Heckers, S., Ohtake, T., Wiley, R. G., Lappi, D. A., Geula, C. & Mesulam, M.-M. (1994) Complete and selective cholinergic denervation of rat neocortex and hippocampus but not amygdala by an immunotoxin against the p75 NGF receptor. *J. Neurosci.* 14, 1271-1289.
- Hendry, I. A., Murphy, M., Hilton, D. J., Nicola, N. A. & Bartlett, P. F. (1992) Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. *J. Neurosci.* 12, 3427-3434.
- Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. & Kishimoto, T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* 63, 1149-1157.
- Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. & Stüber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel chelate absorbent. *Biotechnology* 6, 1321-1325.
- Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N. & Yancopoulos, G. D. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* 69, 1121-1132.
- Kotzbauer, P. T., Lampe, P. A., Estus, S., Milbrandt, J. & Johnson, E. M. Jr (1994) Postnatal development of survival responsiveness in rat sympathetic neurons to leukemia inhibitory factor and ciliary neurotrophic factor. *Neuron* 12, 763-773.
- Jean, L.-F. L. & Murphy, J. R. (1991) Diphtheria toxin receptor-binding domain substitution with interleukin-6: genetic construction and

- interleukin-6 receptor-specific action of a diphtheria toxin-related interleukin-6 fusion protein. *Protein Eng.* 4, 989–994.
- Licemli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lewis, S. E., Rao, M. S., Symes, A. J., Dauer, W. T., Fink, J. F., Landis, S. C. & Hyman, S. E. (1994) Coordinate regulation of choline acetyltransferase, tyrosine hydroxylase, and neuropeptide mRNA by ciliary neurotrophic factor and leukemia inhibitory factor in cultured sympathetic neurons. *J. Neurochem.* 63, 429–437.
- Li, M., Sendtner, M. & Smith, A. (1995) Essential function of LIF receptor in motor neurons. *Nature* 378, 724–727.
- Ludlam, W. H. & Kessler, J. A. (1993) Leukemia inhibitory factor and ciliary neurotrophic factor regulate expression of muscarinic receptors in cultured sympathetic neurons. *Dev. Biol.* 155, 497–506.
- Ludlam, W. H., Zang, Z., McCaeson, K. E., Krause, J. E., Spray, D. C. & Kessler, J. A. (1994) mRNAs encoding muscarinic and substance P receptors in cultured sympathetic neurons are differentially regulated by LIF or CNTF. *Dev. Biol.* 164, 528–539.
- Martinou, J.-C., Martinou, I. & Kato, A. C. (1992) Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons *in vitro*. *Neuron* 8, 737–744.
- Mayer, P., Geissler, K., Ward, M. & Metcalf, D. (1993) Recombinant human leukemia inhibitory factor induces acute phase proteins and raises the blood platelet counts in nonhuman primates. *Blood* 81, 3226–3233.
- Mayer, M., Bhakoo, K. & Noble, M. (1994) Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes *in vitro*. *Development* 120, 143–153.
- Mereau, A., Grey, L., Piquet-Pellorce, C. & Heath, J. K. (1993) Characterization of a binding protein for leukemia inhibitory factor localized in extracellular matrix. *J. Cell Biol.* 122, 713–719.
- Metcalf, D. (1992) Leukemia inhibitory factor – a puzzling polyfunctional regulator. *Growth Factors* 7, 169–173.
- Metcalf, D. & Gearing, D. P. (1989) A fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor (LIF). *Proc. Natl Acad. Sci. USA* 86, 5948–5952.
- Miyajima, A., Hara, T. & Kitamura, T. (1992) Common subunits of cytokine receptors and the functional redundancy of cytokines. *Trends Biochem. Sci.* 17, 378–382.
- Moreau, J. F., Donaldson, D. D., Bennet, F., Witek, Ginnotti, J., Clark, S. C. & Wong, G. G. (1988) Leukemia inhibitory factor is identical to the myeloid factor human interleukin for DA cells. *Nature* 336, 690–692.
- Moskang, J. O., Stenmark, H. & Olsnes, S. (1991) Insertion of diphtheria toxin B fragment into the plasma membrane at low pH. *J. Biol. Chem.* 266, 2652–2659.
- Murphy, M., Reid, K., Brown, M. A. & Bartlett, P. F. (1993) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* 117, 1173–1182.
- Negro, A., Corona, G., Bigon, E., Martini, I., Grandi, C., Skaper, S. D. & Callegaro, L. (1991) Synthesis, purification and characterization of human ciliary neurotrophic factor from *E. coli*. *J. Neurosci. Res.* 29, 251–260.
- Negro, A. & Skaper, S. D. (1995) Production, characterization and cytotoxic properties of a diphtheria toxin-ciliary neurotrophic factor fusion protein. *Protein Eng.* 8, 175–183.
- Owczarek, C. M., Layton, M. J., Metcalf, D., Lock, P., Willson, T. A., Gough, N. M. & Nicola, N. A. (1993) Inter-species chimeras of leukaemia inhibitory factor define a major human receptor-binding determinant. *EMBO J.* 12, 3487–3495.
- Papini, E., Rappuoli, R., Murgia, M. & Montecucco, C. (1993) Cell penetration of diphtheria toxin. *J. Biol. Chem.* 268, 1567–1574.
- Patterson, P. H. (1994) Leukemia inhibitory factor, a cytokine at the interface between neurobiology and immunology. *Proc. Natl Acad. Sci. USA* 91, 7833–7835.
- Patterson, P. H. & Nawa, H. (1993) Neuronal differentiation factors/cytokines and synaptic plasticity. *Cell* 72, 123–137.
- Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P. & Landis, S. C. (1993) Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. *Neuron* 11, 1175–1185.
- Robinson, R. C., Grey, L. M., Staunton, D., Vankelecom, H., Vernallis, A. B., Moreau, J.-F., Stuart, D. I., Heath, J. K. & Jones, E. Y. (1994) The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. *Cell* 77, 1101–1116.
- Ron, D., Bottaro, D. P., Finch, P. W., Morris, D., Rubin, J. S. & Aaronson, S. A. (1993) Expression of biologically active recombinant keratinocyte growth factor. Structure/function analysis of amino-terminal truncation mutants. *J. Biol. Chem.* 268, 2984–2988.
- Rossino, P., Volpe, G., Negro, A., Callegaro, L., Altruda, F., Tarone, G. & Silengo, L. (1995) Ciliary neurotrophic factor-induced gene expression in human neuroblastoma cell lines. *Neurochem. Res.* 20, 675–680.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Sharf, S. J., Higuchi, R., Horn, G. Z., Mullis, B. & Erlich, H. A. (1988) Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 239, 487–491.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratories. Cold Spring Harbor NY.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitor. *Proc. Natl Acad. Sci. USA* 74, 5465–5468.
- Shen, M. M., Skoda, R. C., Cardiff, R. D., Campos-Torres, J., Leder, P. & Ornitz, D. M. (1994) Expression of LIF in transgenic mice results in altered thymic epithelium and apparent interconversion of thymic and lymph node morphologies. *EMBO J.* 13, 1375–1385.
- Skaper, S. D., Facci, L., Milani, D., Leon, A. & Toffano, G. (1990) Culture and use of primary and clonal neural cells. in *Methods in neurosciences* (Conn, P. M., ed.) vol. 2, pp. 17–33. Academic Press. San Diego.
- Squinto, S. P., Aldrich, T. H., Lindsay, R. M., Morrissey, D. M., Panayotatos, N., Bianco, S. M., Furth, M. E. & Yancopoulos, G. D. (1990) Identification of functional receptors for ciliary neurotrophic factor on neuronal cell lines and primary neurons. *Neuron* 5, 757–766.
- Stahl, N. & Yancopoulos, G. D. (1993) The alphas, betas, and kinases of cytokine receptor complexes. *Cell* 74, 587–590.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60–89.
- Taga, T. & Kishimoto, T. (1992) Cytokine receptors and signal transduction. *FASEB J.* 6, 3387–3396.
- Tarroni, P., Rubbioli, F., Chini, B., Zwart, R., Ootgiesen, M., Sher, E. & Clementi, F. (1992) Neuronal type nicotinic receptor in human neuroblastoma and small cell lung carcinoma cell lines. *FEBS Lett.* 312, 66–70.
- Tomida, M., Yamamoto-Yamaguchi, Y. & Hozumi, M. (1984) Purification of a factor inducing differentiation of mouse myeloid leukemic M1 cells from conditioned medium of mouse fibroblast L929 cells. *J. Biol. Chem.* 259, 10978–10982.
- Waring, P. M., Wycherley, K., Cary, D., Nicola, N. & Metcalf, D. (1992) Leukemia inhibitory factor levels are elevated in septic shock and various inflammatory body fluids. *J. Clin. Invest.* 90, 2031–2037.
- Wiley, R. G. (1992) Neural lesioning with ribosome-inactivating proteins: suicide transport and immunolesioning. *Trends Neurosci.* 15, 285–290.
- Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. & Gough, N. M. (1988) Myeloid leukemia inhibitory factor maintains the development potential of embryonic stem cells. *Nature* 336, 684–687.
- Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J. & Patterson, P. H. (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* 246, 1412–1416.
- Yoshikawa, K., Williams, C. & Sabol, S. L. (1984) Rat brain proenkephalin mRNA. cDNA cloning, primary structure, and distribution in the central nervous system. *J. Biol. Chem.* 259, 14301–14308.